DETERMINING FACTORS INFLUENCING NUCLEAR ENVELOPE AND NUCLEAR PORE COMPLEX STRUCTURE

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Determining Factors Influencing Nuclear Envelope and Nuclear Pore Complex Structure. (May 2013)

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The cell's nuclear envelope (NE) has pores that are stabilized by nuclear pore complexes (NPC), large proteinaceous structures whose function is to mediate transport between the nucleus and cytoplasm. Although the transport process is well studied, the mechanism of NPC assembly from its protein constituents (nucleoporins) is less understood. To investigate NPC biogenesis, I investigated mutants that result in defective NPCs in *Saccharomyces cerevisiae.*

First, I examined mutants in the GPI anchor pathway (*gpi1*) that resulted in mislocalized nucleoporins by testing two models: *gpi1* mutants cause either misregulation of N-linked glycosylation or alter membrane properties. To test the models, I combined *gpi1* mutants with a nucleoporin mutant that is susceptible to disruption of glycosylation or with mutants in membrane bending proteins. Select double mutant of each class rescued the growth phenotype of the single mutants. These results indicate that both of the models play a role in NPC assembly. Secondly, we found the proteasome, a complex responsible for degrading proteins is involved in NPC assembly. In order to further investigate interactions between the NPC and the proteasome, I combined the proteasomal mutant with 3 classes of **n**uclear **p**ore **a**ssembly (*npa*) mutants to test

for synergistic interactions. Positive interactions were observed as the proteasome mutant rescued a temperature sensitive *npa* mutant providing further evidence for the role of the proteasome in NPC assembly

DEDICATION

I would like to dedicate this manuscript to my parents.

ACKNOWLEDGEMENTS

I would like to thank Dr.Kathryn Ryan for all her help and invaluable guidance since the start of my research with her in Spring 2012. Her constant patience and understanding allowed me to develop new ideas not only in the lab, but also for the future. I would also like to acknowledge all the members of Ryan Lab for helpful discussions.

NOMENCLATURE

NPC	Nuclear Pore complex
GPI	Glylcosylphosphatidylinositol
Nup	Nucleoporin
INM	Inner nuclear membrane
ONM	Outer nuclear membrane
NE	Nuclear Envelope
POM	Pore outer membrane
Kaps	karyopherins
GTP	Guanosine-5’ triphosphate
GlcNAc-PI	N-acetylglucosamine phosphatidylinositol
GlcNAC	N-acetylglucosamine
UDP-GlcNAc	Uridine diphosphate N-acetylglucosamine
ER	Endoplasmic Reticulum
RTN	Reticulon
npa	nuclear pore assembly

CHAPTER I

INTRODUCTION

The eukaryotic cell is divided into membrane enclosed organelles that enable efficient performance of cellular tasks in specialized microenvironments. The nucleus of the cell is one of these specialized compartments that houses the genome and is separated from the cytoplasmic contents of the cell by a double membrane structure called the nuclear envelope (NE).

The NE is composed of two distinct lipid bilayers, the inner nuclear membrane (INM) and outer nuclear membrane (ONM) that bend sharply and fuse together at multiple locations resulting in cylindrical fissures called nuclear pores. These nuclear pores serve as sites for the assembly of one of the largest and most complex proteinaceous assemblies in the cell called the nuclear pore complexes (NPC), whose primary function is to facilitate transport of macromolecules between the nucleoplasm and the cytoplasm.

The Nuclear pore complex.

The *Saccharomyces cervisiae* NPC is about 40MDa (Hoelz, Debler et al. 2011) and is composed of approximately 30 proteins (nucleoporins) that are present in multiple copies that result in about 500-1000 protein molecules in a fully intact NPC (Hoelz, Debler et al. 2011).The NPC structure is well conserved across vertebrates and invertebrates. It consists of two coaxial rings, connected by eight symmetrical spokes that lie perpendicular to the plane of the NE. These spokes come together and form the coaxial inner and outer membrane rings, forming a central channel that serves as a transport channel (Strambio-De-Castillia, Niepel et al. 2010). Two sets

of fibrils are present at the ends of these rings, with one set extending out into the cytoplasm and the other set forming a basket like structure on the nucleoplasmic side (Aitchison and Rout 2012).

The nucleoporin proteins (nups) are divided into four categories: Pore membrane protein (POMs) that span the pore membrane and function to anchor the NPC to the NE, core scaffold nups that make up the inner and outer rings, phenylalanine-glycine (FG) nups that make up about 11 of the 30 nups that are present along the inner surface of the spokes and the fibrils extending into both the nucleoplasm and the cytoplasm and linker nups that help anchor the FG nups. These nucleoporins are distributed into particular sub-structural domains in the NPC (Strambio-De-Castillia, Niepel et al. 2010) and thus form a massive proteinaceous complex that is embedded in the NE.

Function of the NPC

Transport between the nucleus and the cytoplasm is essential as various proteins and cargo molecules need to be shuttled back and forth. The principal function of the NPC is to facilitate transport of these macromolecules by acting as a highly selective barrier. Molecules that are <40 kDa can freely diffuse across the NPC but larger proteins and mRNA trafficking is controlled by active transport that requires transport signals and nuclear transport factors. The FG sites present in the NPC serve as binding sites for transport factors called karyopherins (Kaps). Two of karyopherins α-Kap and β- Kap play a role in mediating transport. β- Kap, associates with the NLS motifs present on the cargo molecules and with the help of an adaptor molecule α-Kap, it binds to the FG domain of the NPC. The cargo bound by the transport factor shuttles through the

NPC into the nucleus. Upon reaching its destination, the complex dissociates with the help of a small GTPase called Ran

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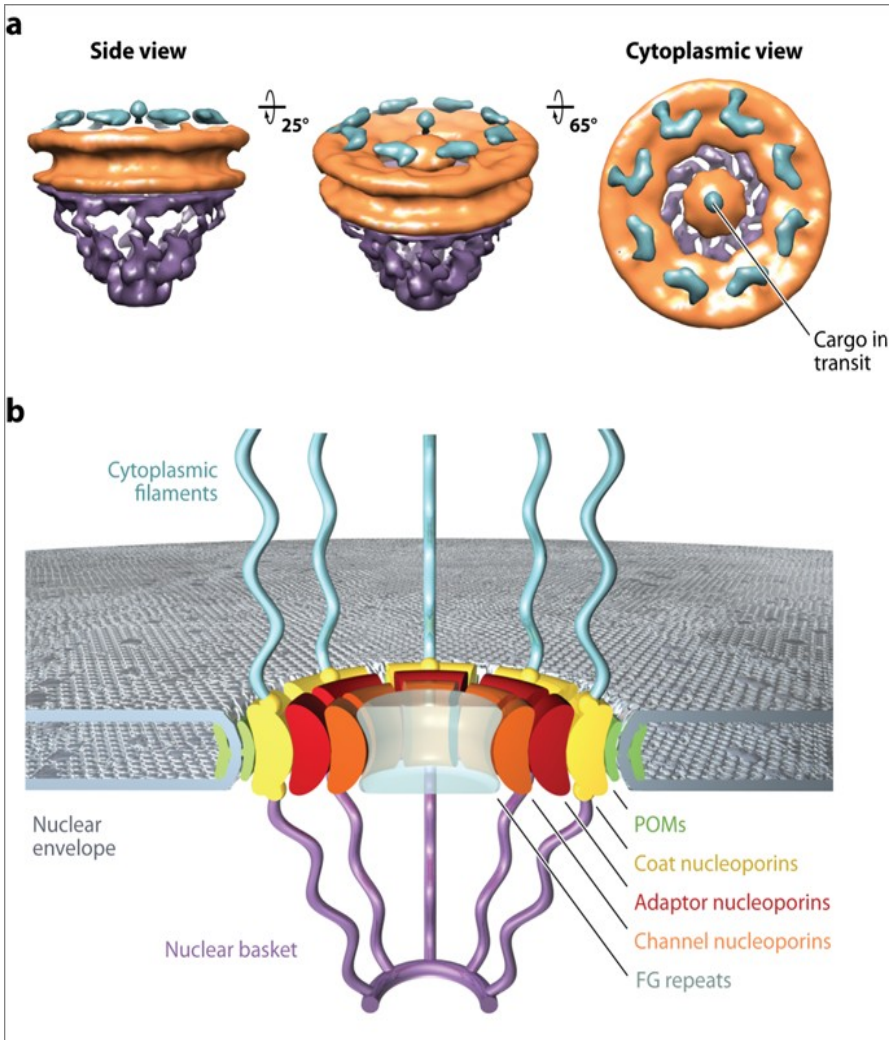

Figure 1. Overall structure of the NPC. (a) Cryo-electron tomographic reconstruction of the NPC. (b) Schematic model of the NPC. (Hoelz A. et al. 2011)

Ran exists in two confirmations: a GTP bound state that is present at high concentrations in the nucleus (maintained by Rcc1 (Prp20 in yeast), a Ran guanyl nucleotide exchange factor) and a

GDP bound form (maintained by RanGAP (Rna1 in yeast), a GTPase-activating protein) localized to the cytoplasm. When the importin bound cargo enters the nucleus, RanGTP promotes the release of the cargo bound transport factor by binding to the Kaps. Nuclear export is mediated through Ran-GTP bound to an exportin that in turn binds to the export cargo. This complex travels back through the NPC to the cytoplasm where the GTP is hydrolyzed resulting in dissociation of the complex .

mRNA that is being synthesized in the nucleus eventually has to be shuttled to the cytoplasm so that it can be translated by the ribosome. This transport is mediated by the NPC but it is independent of Kaps and Ran (Santos-Rosa, Moreno et al. 1998,Katahira, Strasser et al. 1999). The mRNA is packaged into ribonucleotide (RNP) particles that eventually associate with non-kap transport factors which help the mRNA to translocate through the NPC.

NPC assembly

NPC's are complicated structures that have different domains consisting of about 500-1000 proteins that need to be assembled into the NE to facilitate transport across the nucleus and the cytoplasm. NPC biogenesis is linked to cell cycle progression and the breakdown of the NE. In higher metazoans, the NE breaks down during mitosis and is retracted back into the ER so that the nucleus can undergo proper division. At this point the NPC's have to disassemble from the ER and be portioned into vesicles. Some of the nucleoporins associate with the chromatin in the nucleus and form a pre-pore assembly complex (Walther, Alves et al. 2003); (Walther, Askjaer et al. 2003). Once the nucleus has successfully divided, the NE reforms from the ER and the pre-

pore assembly acts as an anchor for the rest of the nucleoporins to come assemble and form a mature NPC.

However, yeast undergo a "closed" mitosis where the NE never breaks down, implying that NPCs have to be inserted into an intact NE. Experiments conducted have shown that NPC assembly is continuous with ~70 NPCs present after mitosis to ~140 NPCs present at anaphase (Winey, Yarar et al. 1997). This assembly process is still unclear and it comprises the main focus of my research.

Work characterizing this assembly process has led to several breakthroughs in understanding the mechanism. A possible theory on assembly process was that NPC subcomponents precursors were accumulated in the NE which formed a scaffolding structure nucleoporins. Research conducted by D'Angelo et al (D'Angelo, Anderson et al. 2006) has shown that NPC assembly into an intact NE can be done independently of pre-existing NPCs, and the assembly happens from both sides of the NE. Mutant analysis in components of the Ran cycle (Ran, RanGEF, RanGAP, NTF2 and Kap95 all have defective NPC assembly phenotypes with mislocalized nucleoporins indicating that the Ran cycle is necessary for proper NPC assembly (Ryan, Zhou et al. 2007, Ryan, McCaffery et al. 2003).

Recent work has also implicated the necessity of membrane bending reticulon proteins (RTNs) and in NPC assembly. Reticulon proteins function to stabilize pore curvature by forming hairpin like structures that wedge themselves into a leaflet of the bilayer (Oertle and Schwab 2003, Shibata, Voss et al. 2008). Deletion studies have indicated that these mutants have NPC

clustering phenotypes, nuclear import defects and synthetic lethality when combined with certain nucleoporins (Dawson, Lazarus et al. 2009). Membrane proteins that are involved in lipid homeostasis (Brr6, Apq12 and Brl1) also have defective NPC assembly phenotypes when they are perturbed implying that proper INM and ONM fusion is necessary for NPC biogenesis (de Bruyn Kops and Guthrie 2001).

To gain further understanding in the NPC assembly process, mutation analysis of key genes that play a role in NPC assembly is necessary. A previous study that utilized Green Fluorescence Protein (GFP) tagged nucleoporins to screen for NPC defects identified mutants with defective NPC assembly (Ryan and Wente 2002). The results from the screen showed that genes in the Glycosylphosphatidylinositol (GPI) anchor pathway play a role in NPC assembly (Ryan Lab, unpublished).

GPI anchors

Post-translational modification of proteins serves to increase the functionality that enable them to contribute to various biological processes. One of these modifications is adding a GPI anchor to the C termini of proteins in order to help them anchor to the cell wall. GPI anchors are synthesized in the Endoplasmic Reticulum (ER) in a multi step pathway involving ~28 proteins (Orlean and Menon 2007) (see Fig 2). Gpi1 is a protein involved in the synthesis of N-acetylglucosamine phosphatidylinositol (GlcNAc-PI), the first key intermediate in the synthesis of GPI anchors by initiating the transfer of N-acetylglucosamine (GlcNAC) from uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) to phosphatidylinositol. It has 6 predicted transmembrane domains and is localized to the ER membrane. Point mutations in *GPI1* that

induce premature stop codons at positions 212 and 536 result in truncated Gpi1 proteins that have a severe NPC assembly defect phenotype.

In order to investigate why *gpi1* mutants have these defects, I tested two models:

1) Misregulation of N-linked Glycosylation causes NPC assembly defects in *gpi1* mutants

UDP-GlcNAc is a nucleotide sugar that is a common precursor for N-linked glycosylation, chitin and GPI anchor synthesis pathways. If GPI anchor biosynthesis is blocked at the Gpi1 stage, there is a tremendous increase in the amount of chitin in the cell (Ryan Lab data, unpublished); however it is unclear if there is mis-regulation of N-linked glycosylation. Previous work by Belanger et al. (Belanger, Gupta et al. 2005) has implicated the role of glycosylation in NPC function. Pom152 is a trans-membrane nucleoporin whose C-terminal segment is glycosylated (Tcheperegine, Marelli et al. 1999).

When the glycosylation domains of this protein are perturbed and combined with a *nup1*Δ, a nucleoporin mutant that has a temperature sensitive growth phenotype and nuclear import

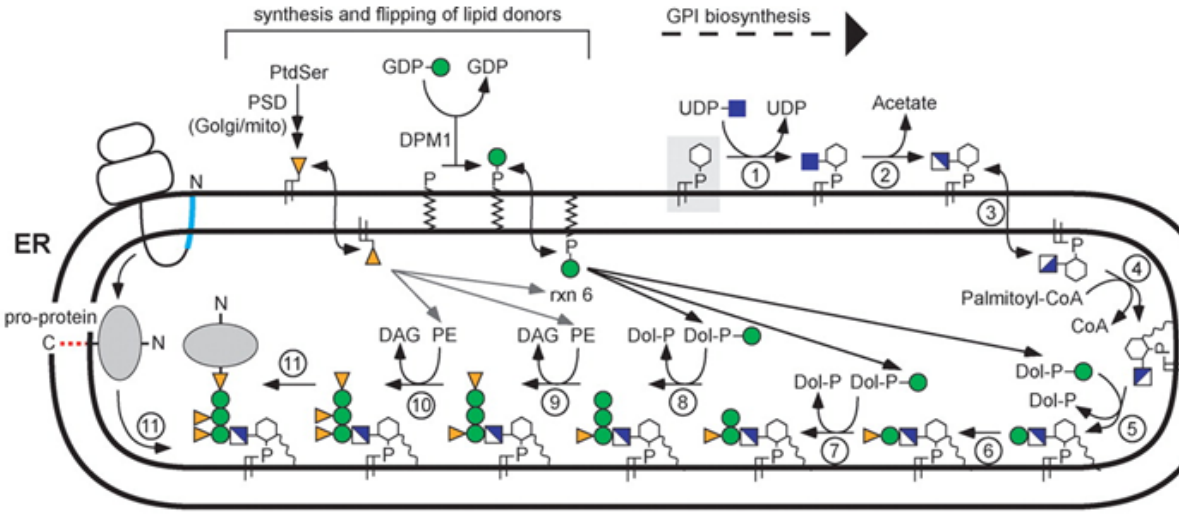

Figure 2. General scheme for GPI biosynthesis in the ER of yeast and mammals. (Orlean, P. and A. K. Menon 2007).

defects, synergistic interactions were observed as the *pom152* mutant rescued the *nup1*Δ defects. Other evidence includes the deletion of a mannosyltransferase (ALG12) suppressing the *nup1*Δ defects and synergistic interactions using chemical inhibitors affecting glycosylation rescuing *nup82*Δ and *nup1*Δ defects (Belanger, Gupta et al. 2005). Since *gpi1* mutants have similar defective NPC phenotypes as the *nup1*Δ, I combined *gpi1* mutants with *nup100*Δ, a nucleoporin that has been shown to relieve *nup1*Δ defects.

2) Gpi1 mutants alter membrane properties that result in defective NPC assembly.

An alternative model as to how *gpi1* mutants affect NPC assembly is that the mutants alter membrane properties. To test if changes in the membrane properties are the reason for NPC defects, I will examine mutants in *RTN1* and *RTN2*. Rtn1 and Rtn2 are membrane shaping proteins with wedge shaped conformations that insert into the membrane, forcing a degree of curvature. If changes in the membrane properties are the reason for NPC defects, then mutations in *rtn1* and *rtn2* may have a synergistic effect of enhancing or suppressing the defects in *gpi1* mutant.

Determine interactions between Proteasome and Nuclear pore complex

The proteasome is a huge protein complex that serves to degrade various ubiquitin tagged protein targets. The catalytic activity of the proteasome is carried out by ATPase subunits that function in substrate unfolding. Mutations in *RPT6*, one of the ATPases of the proteasome have been shown to disrupt nucleoporin assembly in NPCs, causing nups to mislocalize and cluster at various regions in NE (Ryan Lab, unpublished). In order to further test the role of the proteasome in NPC assembly, I combined the *rpt6* mutant with 3 different classes of *npa* mutants: clustering

mutants: *nup120* and *nup133*, nuclear transport factor mutants: *kap95* and *rna1* and finally the GPI anchor mutants: *gab1*, *gpi1-s212* and *gpi1-s537*. Testing these combinations for synergistic interactions has the potential to provide more insight into the role of the proteasome in NPC assembly.

CHAPTER II

MATERIALS AND METHODS

Growth of yeast strains

Yeast were grown in YPD (yeast extract, peptone and 2% glucose) at room temperature unless otherwise stated.

Spot dilution assay

Strains were grown to early log phase at room temperature. 5 x 10^5 cells were harvested, serially diluted 5-fold and spotted on to the appropriate plates.

Generating yeast strains:

Transformation of Yeast

Transformations were performed using the lithium acetate method as described in (Ito, Fukuda et al. 1983). 50ml of culture was growth with shaking to OD_{600} = ~0.5 to 0.7 (must be in log phase). Cells were washed with Lithium Acetate – TE solution and then transformations were setup with the appropriate miniprep DNA, carrier DNA and DMSO. 40% PEG-4000 in LiAC-TE was used to facilitate efficient transformation. After incubation (30C for 30 mins) and heat shock (42C for 15 mins), cells were washed with TE and plated on selective media.

Solutions for transformations:

LiAC-TE Soln = Filter sterilized from combination of 10X stocks

10X TE = 0.1M Tris HCL, 0.01M EDTA pH 7.5

10X LiAC = 1 M LiAC pH 7.5 with diluted acetic acid

40% PEG 4000 LiAC-TE soln : add PEG to appropriate 10X stocks LiAC and 10x TE. Filter sterilized

Carrier DNA: Made from salon sperm or calf thymus DNA as per (Schiestl, Gietz 1989)

Yeast mating protocol

Crosses were carried out with an appropriate *MAT***a** and *MAT***α** strains to generate a diploid *MAT***a/α**. After selection for diploids, the strains were placed in sporulation media where four haploid spores were produced from each meiosis. The ascus wall was digested using a 1:1 mixture of Sorbitol and Zymolyase and the tetrad was seperated and germinated. After the tetrads formed colonies, they were genotyped by replica plating to various selective media to test for the appropriate markers.

Microscopy

GFP was used to visualize live cells using an Olympus microscope with a 100x oil-immersion objective.

Cloning protocol for CDC31

Gateway cloning, from Invitrogen was used for constructing a CDC31 expression plasmid. PCR with genomic DNA as a template and *CDC31* oligos was used to generate an attB-flankedPCR product. A BP reaction was carried out using a donor vector combined with the attB-flanked PCR product to generate a Gateway entry clone. Electro competent *E.coli* cells were transformed using electroporation and incubated at 37C for 45 minutes and plated on LB+Tetracycline (50µg/ml). Positive colonies were selected and grown overnight in 2ml cultures. Miniprep of the E.coli was performed using QIAprep Spin Miniprep KIT (250) to isolate the Gateway entry clone. The CDC31 gene was then transferred to a Gateway Destination vector and the transformation of *E.coli* cells was followed just as indicated before. The cells were plated on LB+Carbinicillin (50µg/ml) plates and positive colonies were selected and grown overnight in 2ml cultures. The Miniprep of the E.coli cells after the LR reaction was performed using the alkaline lysis method. The resulting plasmid was verified using a double restriction digest using

Xba and HindIII enzymes. The plasmid was further verified through sequencing using the Big Dye Kit according to the manufactures instructions.

Table 1. Strain list

Strain name	Genotype
YGS 73	*GFP-nic96:HIS3 ade2-1::ADE2 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*
KRY 1328	*GFP-nic96:HIS3 ade2-1::ADE2 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 gpi1-S212*
KRY 1335	*nic96-GFP:HIS3 ade2-1::ADE2 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 rtn1Δ::KAN gpi1-S212*
KRY 1326	*nic96-GFP:HIS3 ade2-1::ADE2 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 rtn1Δ::KAN*
KRY 1360	*trp1-1 ura3-1his3-11,15 leu2-3,112 can1-100 GFP-nic96:HIS3 gpi1-S537:HPH*
KRY 1361	*nic96-GFP:HIS3 ade2-1::ADE2 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 rtn1Δ::KAN gpi1-S537:HPH*
KRY 1329	*nic96-GFP:HIS3 ade2-1::ADE2 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 rtnΔ2::KAN gpi1-S212*
KRY 1327	*nic96-GFP:HIS3 ade2-1::ADE2 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 rtnΔ2::KAN*
KRY 1364	*nic96-GFP:HIS3 ade2-1::ADE2 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 rtn2Δ::KAN gpi1-S537:HPH*
KRY 1331	*nic96-GFP:HIS3 ade2-1::ADE2 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 nup100::KAN gpi1-S212*
KRY 1308	*nup100Δ::KAN nic96-GFP:HIS3 ade2-1::ADE2 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*
KRY 1359	*trp1-1 ura3-1his3-11,15 leu2-3,112 can1-100 GFP-nic96:HIS3 gpi1-S537:HPH nup100Δ:KAN*
YGS 178	*can1Δ ::STE2pr-Sp_his5 lyp1Δ ::STE3pr-LEU2 his3Δ leu2Δ0 ura3Δ0*
KRY 922	*can1Δ ::STE2pr-Sp_his5 lyp1Δ ::STE3pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0 nic96-GFP:HPH rpt6/npa17:NAT*
KRY 992	*can1Δ ::STE2pr-Sp_his5? lyp1Δ ::STE3pr-LEU2+ his3Δ1 leu2Δ0 ura3Δ0 nic96-GFP:HPH rpt6/npa17:NAT rpt6::kanR-tet07-TATA URA3::CMV-tTA MATa met15-0?*

KRY 991	*can1Δ ::STE2pr-Sp_his5? lyp1Δ ::STE3pr-LEU2+ his3Δ1 leu2Δ0 ura3Δ0 nic96-GFP:HPH rpt6::kanR-tet07-TATA URA3::CMV-tTA MATa met15-0?*
YGS 121	*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*
KRY 1318	*kap95-E126K nic96-GFP:HPH his3 leu2 ura3 trp1-1 met15-0? can1-100?*
KRY 1319	*kap95-E126K rpt6::kanR-tet07-TATA URA3::CMV-tTA nic96-GFP:HPH his3 leu2 ura3 met15-0? can1-100?*
KRY 1404	*His3Δ1 leu2Δ0 ura3Δ0 met15Δ0 nic96-GFP:HPH URA3::CMV-tTA*
KRY 1409	*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 nup133Δ::KAN nic96-GFP:HPH*
KRY 1295	*nup133Δ::KAN rpt6::kanR-tet07-TATA URA3::CMV-tTA nic96-GFP:HPH his3Δ1 leu2Δ0 met15Δ0? ura3Δ0*
KRY 1332	*nup120Δ::HIS3 nic96-GFP:HPH his3 leu2 ura3 can1? met15-0?*
KRY 1334	*nup120Δ::HIS3 rpt6::kanR-tet07-TATA URA3::CMV-tTA nic96-GFP:HPH his3 leu2 ura3 can1? met15-0*
KRY 89	*trp1-1 ura3-1 his3-11,15 leu2-3,112 can1-100 rna1-S116F*
KRY 1418	*nic96-GFP:HPH rpt6::kanR-tet07-TATA URA3::CMV-tTA rna1-S116F his- leu-*
KRY 1417	*rpt6::kanR-tet07-TATA URA3::CMV-tTA nic96-GFP:HPH gab1-1 his- leu-*
KRY 1356	*his3 leu2 lys2 trp1 ura3 nic96-GFP:HPH gab1-1*
KRY 1414	*nic96-GFP:HPH rpt6::kanR-tet07-TATA URA3::CMV-tTA gpi1-S212 his- leu- trp-*
KRY 1419	*nic96-GFP:HPH rpt6::kanR-tet07-TATA URA3::CMV-tTA gpi1-S537:HPH leu-*

CHAPTER III

RESULTS

Deletion of *nup100* suppress defects in *gpi1* mutants

In order to investigate if *gpi1* mutants cause misregulation of N-linked glycosylation, *NUP100* was deleted in strains that had point mutations in *gpi1,* which induce premature stop codons at amino acid positions 212 and 537, leading to truncated Gpi1 proteins. These *gpi1* mutants exhibit temperature sensitivity at 34°. Strains were assayed for growth on rich media at 15°, 23°, 30°and 34° by 5-fold serial dilutions. Positive interactions were observed at 15° with the *gpi1-S537/nup100*Δ double mutant rescuing the cold sensitive growth phenotype of both the *nup100*Δ and the *gpi1-S537* single mutants and the *gpi1-s212/nup100*Δ double mutant partially rescuing the *nup100*Δ mutant (See Fig. 3.) The *gpi1-S537/nup100*Δ also presented enhanced sickness at 23° and 30° when compared to either of the single mutants. These results indicate that misregulation of N-linked glycosylation has a role in *gpi1* mutants.

	15°	**23°**	**30°**	**34°**
WT	0	0	0	0
gpi1-S212	0	0	0/-	-
gpi1-S212/nup100Δ	0/+	0/-	-	-
nup100Δ	0/- -	0	0	0
gpi1-S537/nup100 Δ	+	0	0/-	-
gpi1-S537	0/-	0	0/-	-

Table 2: *nup100* interactions with *gpi1* mutants. Double mutants were generated and scored for growth against single mutants: "+" indicates rescue, "0/+" indicates partial rescue, "0"

indicates that cells were viable "0/-" indicates enhanced sickness, "0/--" indicates extreme sickness, "-" indicates lethality.

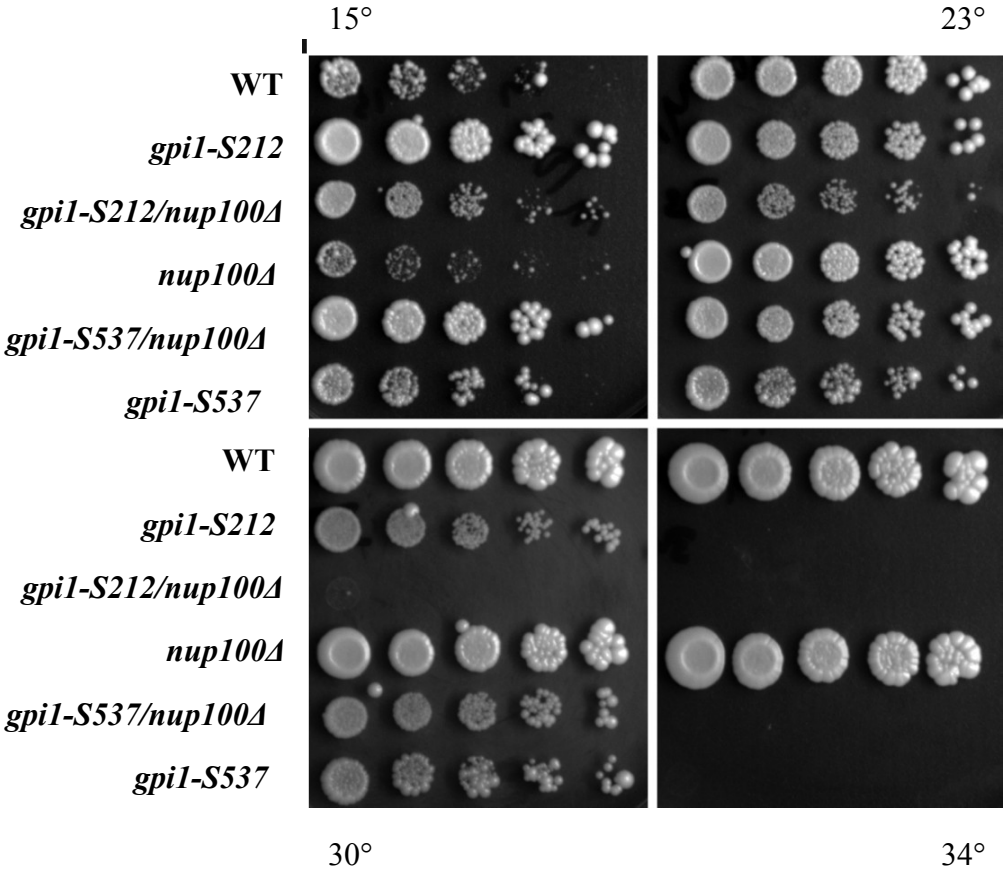

Figure 3. ***nup100*** **interactions with** ***gpi1*** **mutants. 5X serial dilutions on YPD plates**

Altering membrane properties and their effects in *gpi1* mutants

Reticulon proteins are hairpin structures that are responsible for the curvature of the ER. They have also been shown to play a role in NPC assembly. To test if altering membrane properties have synergistic effects on growth, *RTN1* was deleted in strains with mutations in the GPI pathway. Strains were assayed for growth at 15°, 23°, 30°and 34°. The *gpi1-S212/rtn1*Δ double mutant rescued the partial lethality of *rtn* Δ at 15° (See Fig. 4). The growth of this double mutant was better than the wild type at the lower temperature. Synthetically lethal interactions were observed in the *gpi1-S537/rtn1*Δ strain across all temperatures. To further explore the effects of

deleting reticulon proteins in the *gpi1* mutants, *rtn2* was deleted in *gpi1* strains and assayed for growth as before. The results were not dramatic as the double mutants were sicker compared to the single mutants. A faint partial rescue of the *gpi1-S537/rtn2Δ* mutant was seen at 23° when compared to the *gpi1-s537* mutant (See Fig. 5). These results indicate that *gpi1* mutants have altered membrane properties that can be restored by perturbing reticulon proteins.

	15°	**23°**	**30°**	**34°**
WT	0	0	0	0
gpi1-S212	0	0	0	-
gpi1-S212/rtn1Δ	+	0	0/-	-
rtn1Δ	0/-	0	0	0
gpi1-S537/rtn1Δ	-	0/-	0/-	-
gpi1-S537	0/-	0	0/-	-

Table 3: Rtn1 interactions with *gpi1* mutants. Double mutants were generated and scored for growth against single mutants: "+" indicates rescue, "0/+" indicates partial rescue, "0" indicates that cells were viable "0/-" indicates enhanced sickness, "0/--" indicates extreme sickness, "-" indicates lethality.

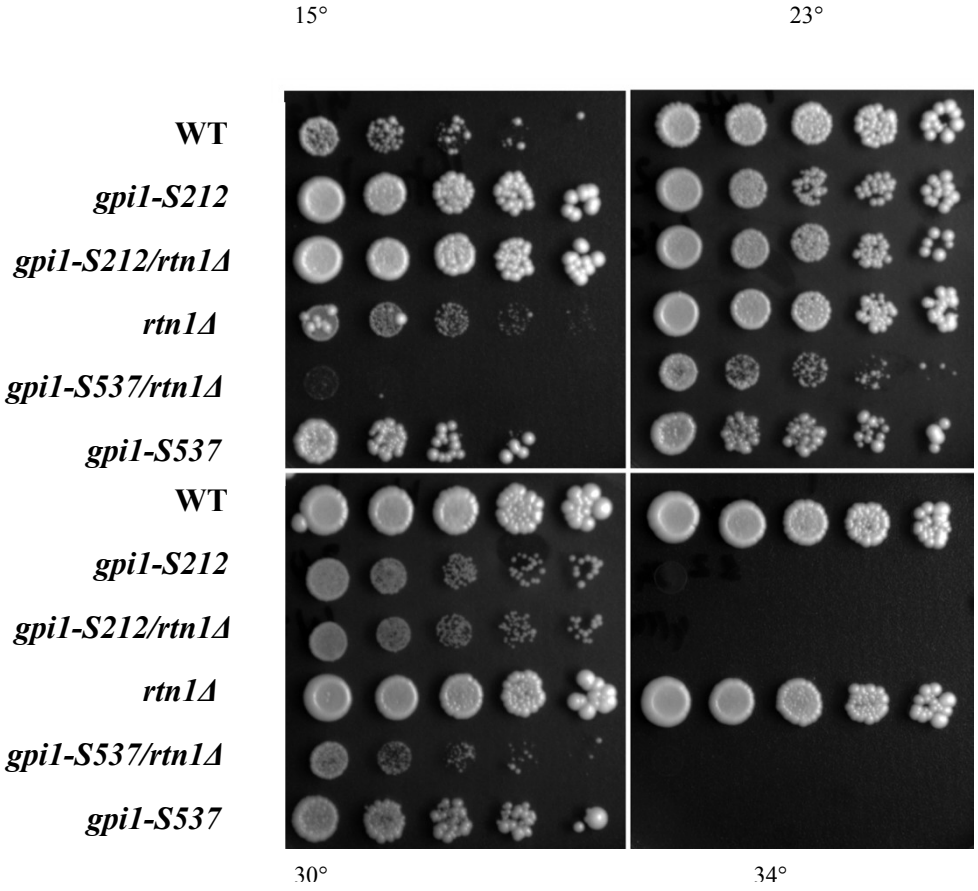

Figure 4. *Gpi1* interactions with *rtn1Δ*. 5X serial dilutions on YPD plates

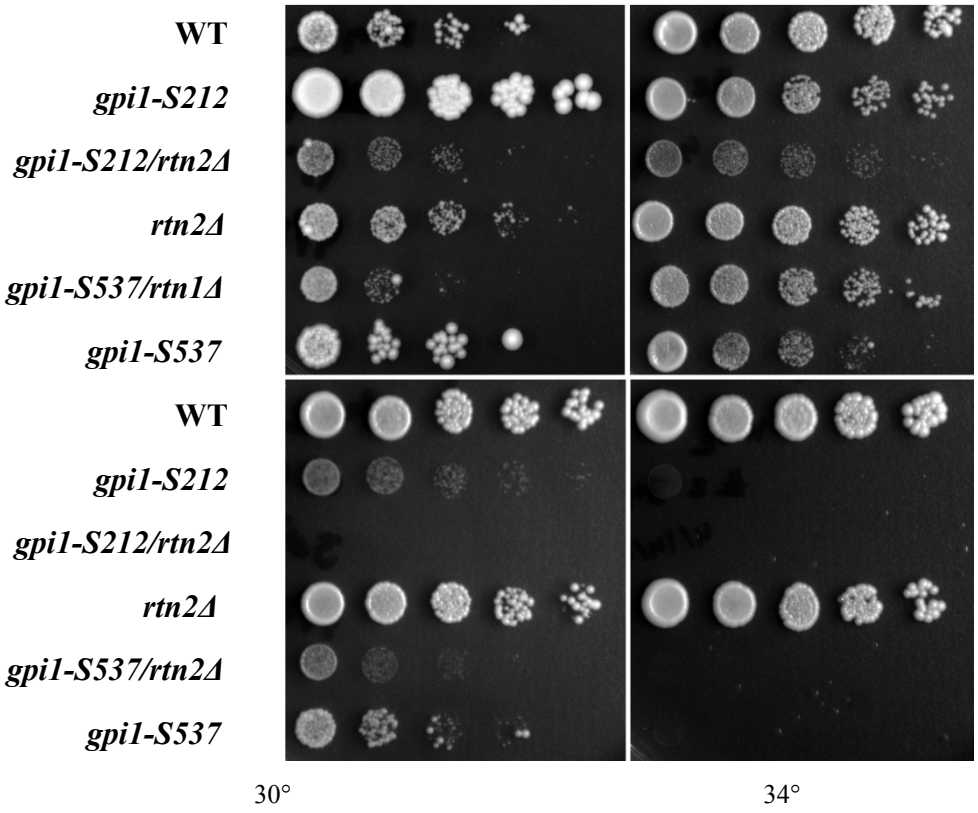

Figure5. *Gpi1* interactions with *rtn2*Δ. 5X serial dilutions on YPD plates

	15°	23°	30°	34°
WT	0	0	0	0
gpi1-S212	0	0	0/-	-
gpi1-S212/rtn2Δ	0	0/-	-	-
rtn2Δ	0	0	0	0
gpi1-S537/rtn2Δ	0/-	0/+ (weak)	0/-	-
gpi1-S537	0/-	0	0/-	-

Table 4: Rtn2 interactions with *gpi1* mutants. Double mutants were generated and scored for growth against single mutants: "+" indicates rescue, "0/+" indicates partial rescue, "0" indicates that cells were viable "0/-" indicates partial sickness, "0/--" indicates extreme sickness, "-" indicates synthetic lethality.

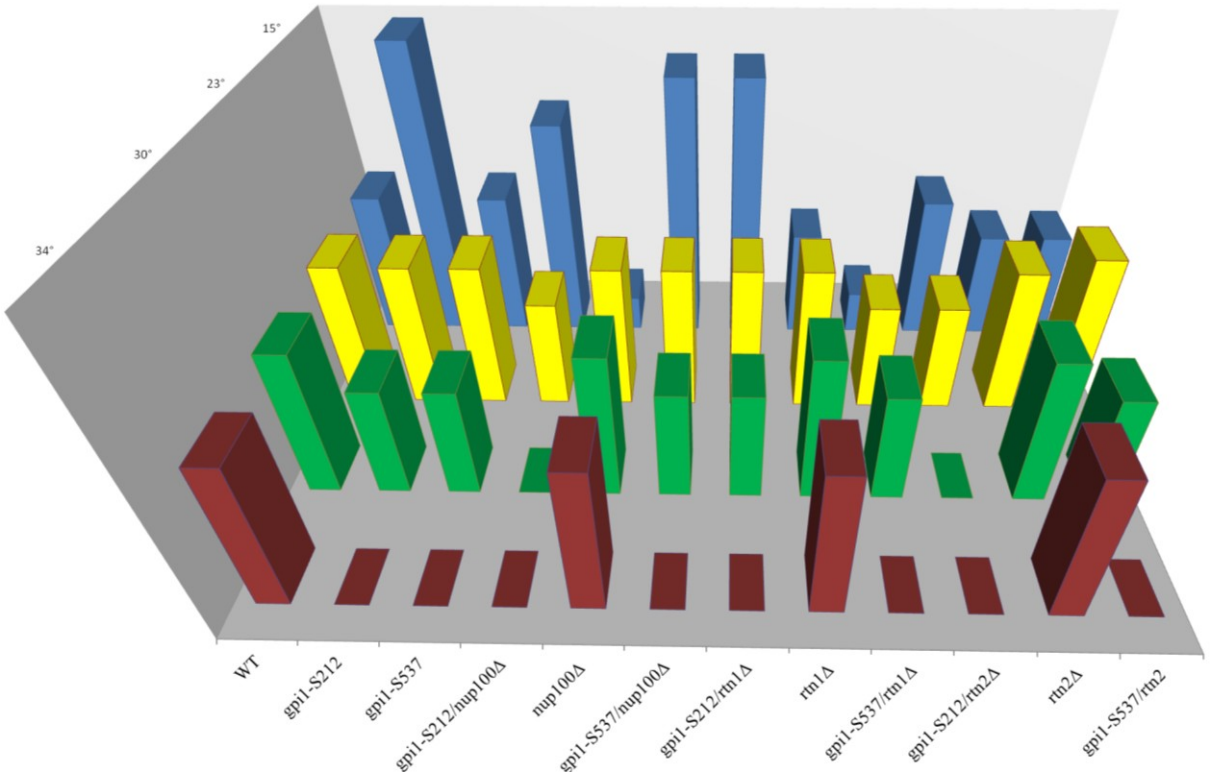

Chart 1 : Summary of GPI1 growth interactions normalized to WT

NPC interactions with the Proteasome

Previous lab data showed that the proteasome is responsible for NPC assembly as disruptions in *rpt6*, an ATPase of the proteasome resulted in a *npa* phenotype. The next sets of experiments performed were to observe the interactions between the *rpt6* mutant in combination with various classes of *npa* mutants to further investigate the role of the proteasome in NPC assembly. Since *RPT6* is an essential gene, mutational analysis by knockout was not possible. In order to bypass this, the endogenous promoter was replaced with a Tet-titratable promoter that allows the expression of the gene to be switched off by the addition of doxycycline. Double mutants were generated and assayed for growth on media containing Doxycycline (10µg/ml) at various temperatures.

Clustering mutant interactions with *rpt6*

	15 Dox+	**15 Dox-**	**23 Dox+**	**23 Dox-**	**30 Dox+**	**30 Dox-**	**34 Dox+**	**34 Dox-**
WT	0	0	0	0	0	0	0	0
nup120Δ	0/-	0/-	0/-	0/-	0/-	0/-	-	-
nup120Δ /rpt6	0/-	0/-	0/-	0/-	0/-	0/-	-	-
nup133Δ	0	0	0	0	0	0/--	0	-
nup133/rpt6	-	0	-	0	-	+	-	+
rpt6	-	0	-	0	-	0	-	0

Table 6: *rpt6* interactions with *nup120*Δ and *nup133*Δ mutants. Double mutants were generated and scored for growth against single mutants: "+" indicates rescue, "0/+" indicates partial rescue, "0" indicates that cells were viable "0/-" indicates partial sickness, "0/--" indicates extreme sickness, "-" indicates synthetic lethality.

Nup120 and Nup133 are non-essential nuclear pore proteins, that when disrupted, causes cells to exhibit a NPC clustering phenotype where the NPCs are concentrated in a discrete area of the nuclear envelope instead of being evenly distributed as seen in the WT cells. To test if these mutants have synergistic interactions with the *rpt6* mutant, double mutant strains were generated by deleting the *NUP120* and the *NUP133* genes in the *rpt6* mutants. The strains were assayed for growth at 15°,23°, 30°and 34°. Positive interactions were observed at 30°and 34° where the temperature sensitive *nup133*Δ was rescued by the *rpt6* mutant with the altered promoter (See Fig. 6). However, when *RPT6* expression was eliminated by the addition of Dox, the *nup133*Δ did not rescue at any temperature. No synergistic interactions were observed with the nup120 Δ strains (See Fig. 6).

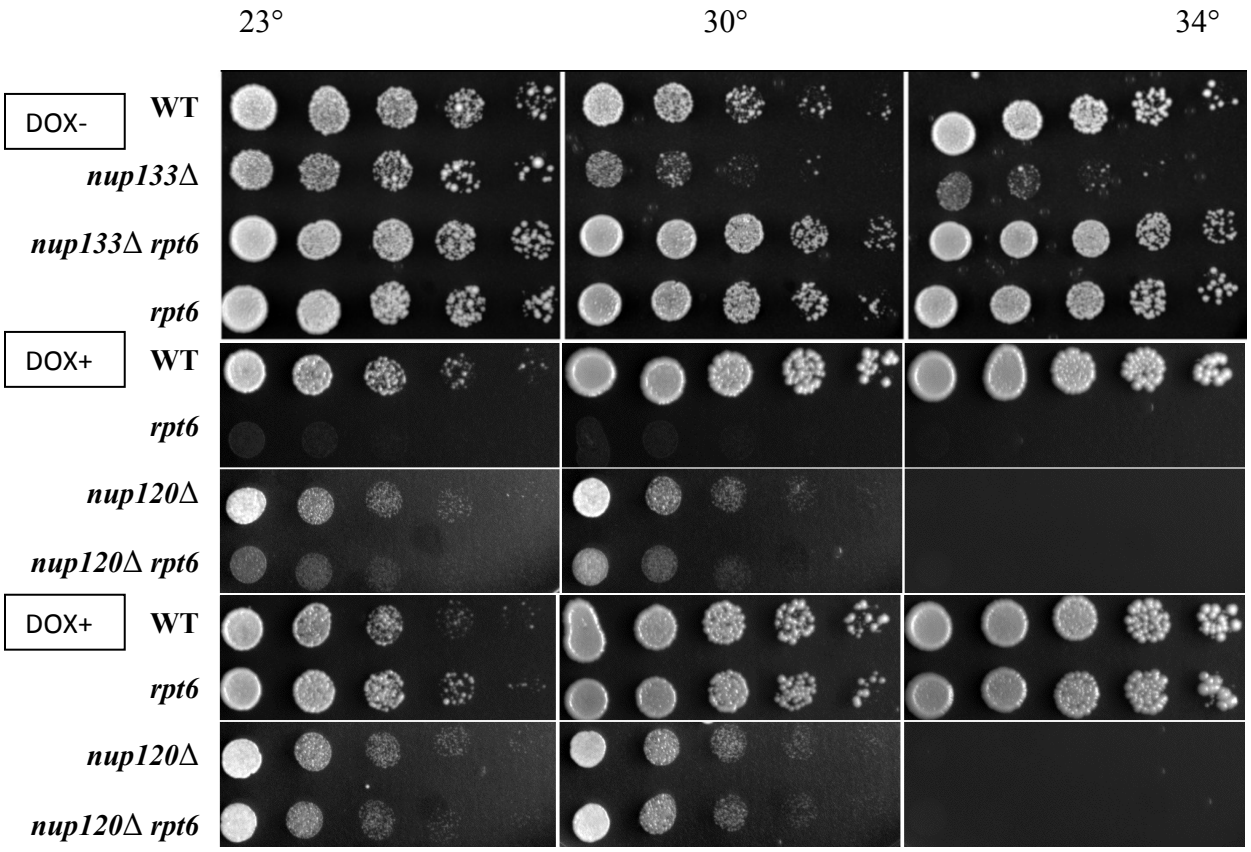

Figure 6. *nup133*Δ and *nup120* Δ interactions with *rpt6*. 5X serial dilutions on YPD plates

Nuclear Transport factors interactions with *rpt6*

A second class of mutants with defective NPCs involves mutants in the nuclear transport factors *kap95* and *rna1*. Both of these mutants have a temperature sensitive phenotype at 34°. Double mutants were generated and assayed for growth on media containing YPD and YPD+ Doxycycline (10ug/ml) at various temperatures. Partial negative interactions were observed in the *kap95 rpt6* double mutant when compared to the single mutants. The double mutant grew worse than either of the singles at 23° and 30° on Dox- plates. The *rna1 rpt6* also showed negative interactions at 34° on Dox-, as the double grew worse than either of the single mutants.

No positive interactions were observed as neither of the nuclear transport factor mutants rescued

	23° Dox+	23° Dox-	30° Dox+	30° Dox-	34° Dox+	34° Dox-
WT	0	0	0	0	0	0
kap95	0	0	0	0	-	-
kap95 /rpt6	-	0/-	-	0/-	-	-
rpt6	-	0	-	0	-	0
rna1	0	0	0	0	0	0
rna1/rpt6	-	0/-	-	0	-	0/-

the *rpt6* mutant or vice versa.

Table 6: *rpt6* interactions with *kap95*and *rna1* mutants. Double mutants were generated and scored for growth against single mutants: "+" indicates rescue, "0/+" indicates partial rescue, "0" indicates that cells were viable "0/-" indicates partial sickness, "0/--" indicates extreme sickness, "-" indicates synthetic lethality.

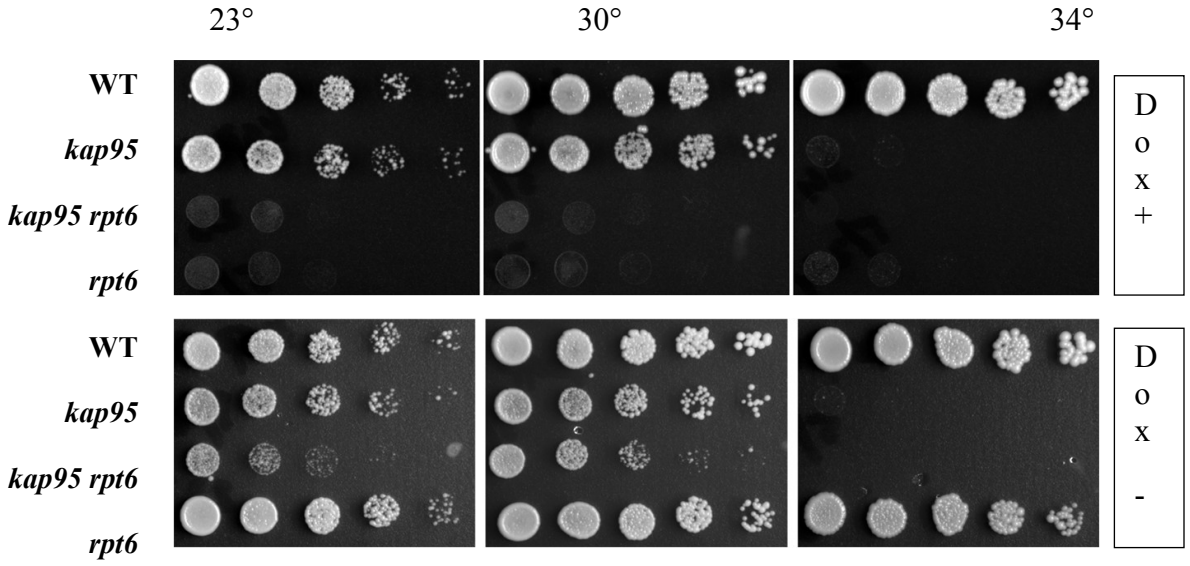

Figure 7. *kap95* interactions with *rpt6*. 5X serial dilutions on YPD and YPD+DOX plates

GPI anchor pathway interactions with rpt6

The last class of NPC mutants tested in combination with *rpt6* mutant were in the GPI anchor pathway. These mutants were assayed for growth at various temperatures. No synergistic interactions were observed as none of the GPI anchor mutants rescued the *rpt6* mutant or vice versa.

	15° Dox+	**15° Dox-**	**23° Dox+**	**23° Dox-**	**30° Dox+**	**30° Dox-**	**34° Dox+**	**34° Dox-**
WT	0	0	0	0	0	0	0	0
gab1	0	0	0	0	0	0	0/-	0/-
gab1/rpt6	-	0/-	-	0/-	-	0	-	-
gpi1-s212	0	0	0	0	0/--	0/-	-	-
gpi1-s212/rpt6	-	0	-	0	-	0/--	-	-
gpi1-s537	0	0	0	0	00/-	0	-	-
gpi1-s537/rpt6	-	0/-	-	0/-	-	--	-	-
rpt6	-	0	-	0	-	0	-	0

Table 7: *rpt6* interactions with *gab1*, *gpi1-s212* and *gpi1-s537* mutants. Double mutants were generated and scored for growth against single mutants: "+" indicates rescue, "0/+" indicates partial rescue, "0" indicates that cells were viable "0/-" indicates partial sickness, "0/--" indicates extreme sickness, "-" indicates synthetic lethality.

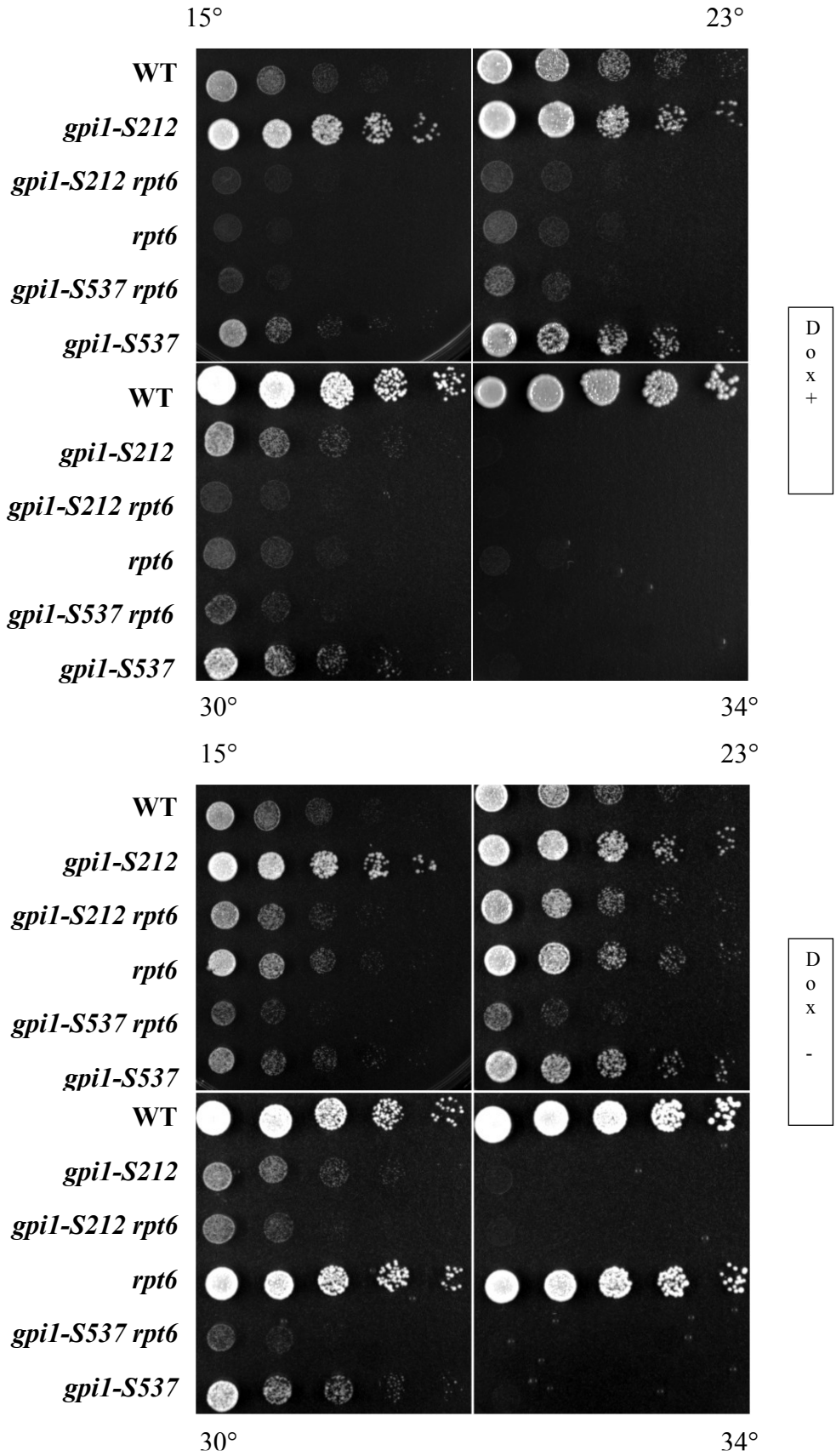

Figure 7. *rpt6* interactions with *gpi1-S212* and *gpi1-S537* . 5X serial dilutions on YPD and YPD+DOX plates

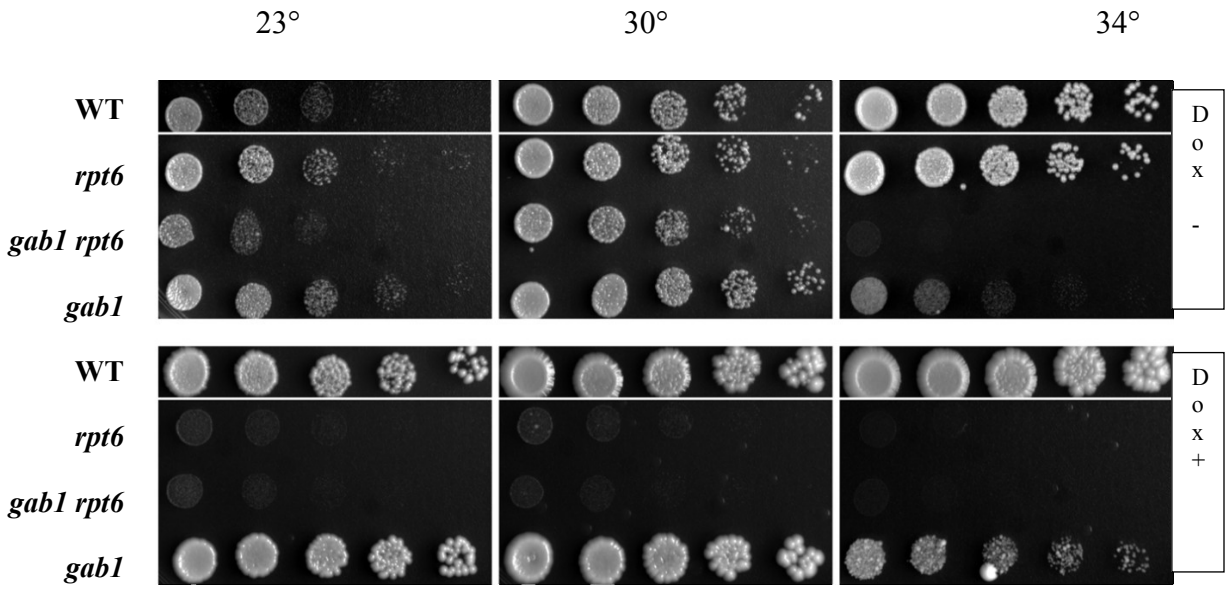

Figure 10. *rpt6* interactions with *gab1* mutants. 5X serial dilutions on YPD and YPD+DOX plates

CHAPTER IV

DISCUSSION

The main focus of my research was to investigate NPC biogenesis by examining mutants that result in defective NPCs. This was done by examining mutants in the GPI anchor pathway and the proteasome.

GPI anchor pathway

First, I examined mutants in the GPI anchor pathway (*gpi1*) that resulted in mislocalized nups by testing two models: *gpi1* mutants cause misregulation of N-linked glycosylation or *gpi1* mutants alter membrane properties. To test the first model, I combined *gpi1* mutants with *nup100*Δ which has shown to relieve defects in a mutant with a similar phenotype to the *gpi1* mutants. To test the second model, I combined *gpi1* with mutants in membrane bending proteins (*rtn1*Δ and *rtn2* Δ). Select double mutant of each class rescued the growth phenotype of the single mutants indicating that both of the models play a role in NPC assembly. The membrane bending synergistic effects of the *gpi1 rtn1*Δ and *gpi1 rtn2* Δ mutants might be a result of altered lipid profiles in the NE. A lipid extraction assay can be performed which would confirm this hypothesis.

Proteasome and NPC

Since the *rpt6* mutant has a severe *npa* phenotype with clustering of nucleoporins at a periphery of the NE along with NPC constituents trailing into the ER, I combined this mutant with various

other *npa* mutants to test to see if these aberrations could be relieved. These interactions would provide insight into factors required for NPC localization, assembly and organization.

When *rpt6* was combined with *nup133*Δ, positive interactions were seen as the altered promoter mutant rescued the temperature sensitive phenotype of the *nup133Δ* mutant. This was interesting, as this interaction was observed on regular media without doxycycline instead of the media with the doxycycline where *rpt6* is completely down regulated. It is important to understand that though this interaction was seen on regular media, the results observed are still significant due to the altered promoter that changes gene expression levels. This altered promoter might have an increased or a decreased affinity for the RNA polymerase; thereby either increasing or decreasing the amount of mRNA produced. The altered mRNA expression levels will translate to the quantity of Rpt6 protein being produced.

In a cell, there is a balance between the amount of a particular protein being produced by the ribosomes and protein turnover mediated by the proteasome. In the altered promoter *rpt6* mutant, the degradation pathway is altered in such a way that there is either excess amount degradation due to increased catalytic activity of the proteasome or there not enough protein turnover due to a defective proteasome. The overall activity of the proteasome is linked to the amount of RPT6 mRNA being produced which is unknown. In order to measure the amount of mRNA produced, a reverse transcription PCR assay can be performed which will provide an estimate of RPT6 being made in the mutant strain versus the WT strain. Based on this, there are two possibilities that can explain why the altered promoter RPT6 can rescue the temperature sensitivity of the *nup133*Δ strain.

Hyperactive Proteasome/ Factor X hypothesis – Proteasomes are localized to the nucleus and the cytosol. The main function of the nuclear proteasome is to degrade oxidative damaged histones, various nuclear proteins. The deletion of *nup133* gene has been shown to result in abnormal mRNA accumulation in the nucleus. Although there is no evidence for this, I propose that Nup133 functions as a transcription factor and affects "factor X" mRNA levels by down regulating its gene expression. In a *nup133Δ* single mutant, this interaction does not exist, resulting in "factor X" mRNA levels being expressed at higher levels than what is observed at WT. However in the *nup133* Δ *rpt6* double mutant, the hyperactive proteasome which is the result of the altered promoter that produces excess RPT6 mRNA degrades "factor X's" mRNA at higher levels, essentially duplicating NUP133's function in "factor X's" gene expression. This would restore balance back to WT levels.

Decreased proteasome activity hypothesis - mRNA is transcribed in the nucleus then exported out into the cytoplasm where it is translated by ribosomes to form a protein product. The turnover of this protein is mediated by the proteasome which has reduced activity in the altered promoter *rpt6* mutant. This leads to an accumulation of the protein in the cytosol as it is not being constantly degraded. The *nup133*Δ causes an accumulation of mRNA in the nucleus. When this mRNA is not exported out to the cytosol, the protein concentration in the cytoplasm is effectively decreased as this mRNA cannot be translated by the ribosomes to form new protein. When *nup133*Δ is combined with an *rpt6* mutant, the abnormal mRNA export defect of the *nup133*Δ is nullified by the defective proteasome function that fails to turn over the existing protein. (See Fig. 11). This model is much more plausible than the previous model.

The key to solving this is dependent on the activity level of the proteasome. The proteasome catalyzes degradation of proteins that have been tagged with a poly-ubiquitin chain. To measure the activity level of the proteasome, ubiquitin can be fused to a β-Gal target that will eventually be directed to the proteasome for degradation. Since β-Gal activity is easily quantifiable, it will be a direct correlation of the level of proteasomal activity and provide evidence for one of the two models.

The new interactions found between these complexes and pathways provide insight into different factors necessary for proper NPC biogenesis.

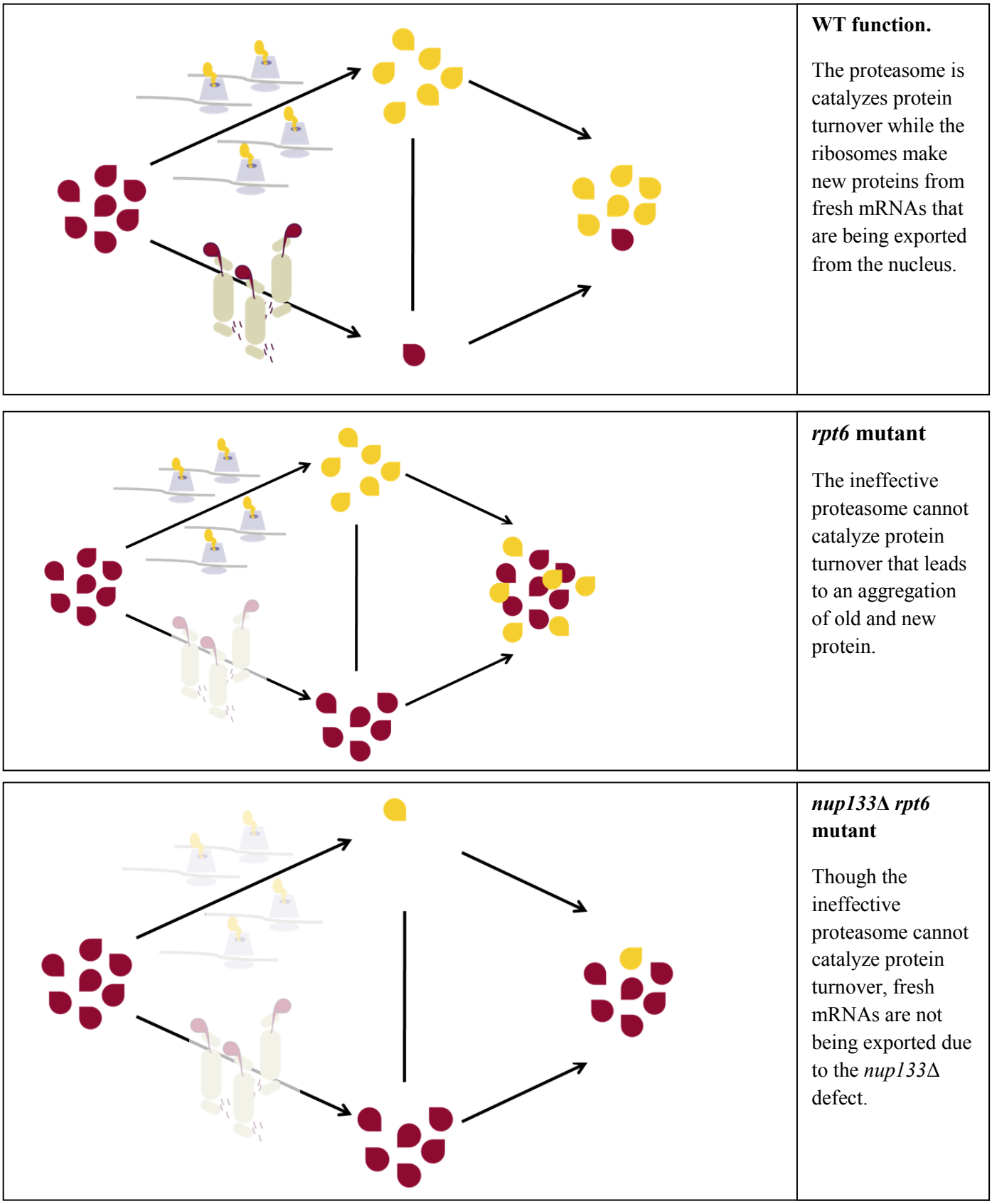

Figure 11. Decreased proteasome hypothesis

REFERENCES

Aitchison, J. D. and M. P. Rout (2012). "The yeast nuclear pore complex and transport through it." Genetics 190(3): 855-883.

Belanger, K. D., A. Gupta, K. M. MacDonald, C. M. Ott, C. A. Hodge, C. M. Cole and L. I. Davis (2005). "Nuclear pore complex function in Saccharomyces cerevisiae is influenced by glycosylation of the transmembrane nucleoporin Pom152p." Genetics 171(3): 935-947.

D'Angelo, M. A., D. J. Anderson, E. Richard and M. W. Hetzer (2006). "Nuclear pores form de novo from both sides of the nuclear envelope." Science 312(5772): 440-443.

Dawson, T. R., M. D. Lazarus, M. W. Hetzer and S. R. Wente (2009). "ER membrane-bending proteins are necessary for de novo nuclear pore formation." J Cell Biol 184(5): 659-675.

de Bruyn Kops, A. and C. Guthrie (2001). "An essential nuclear envelope integral membrane protein, Brr6p, required for nuclear transport." EMBO J 20(15): 4183-4193.

Hoelz, A., E. W. Debler and G. Blobel (2011). "The structure of the nuclear pore complex." Annu Rev Biochem 80: 613-643.

Katahira, J., K. Strasser, A. Podtelejnikov, M. Mann, J. U. Jung and E. Hurt (1999). "The Mex67p-mediated nuclear mRNA export pathway is conserved from yeast to human." EMBO J 18(9): 2593-2609.

Oertle, T. and M. E. Schwab (2003). "Nogo and its paRTNers." Trends Cell Biol 13(4): 187-194.

Orlean, P. and A. K. Menon (2007). "Thematic review series: lipid posttranslational modifications. GPI anchoring of protein in yeast and mammalian cells, or: how we learned to stop worrying and love glycophospholipids." J Lipid Res 48(5): 993-1011.

Rout, M. P. and G. Blobel (1993). "Isolation of the yeast nuclear pore complex." J Cell Biol 123(4): 771-783.

Ryan, K. J., J. M. McCaffery and S. R. Wente (2003). "The Ran GTPase cycle is required for yeast nuclear pore complex assembly." J Cell Biol 160(7): 1041-1053.

Ryan, K. J. and S. R. Wente (2002). "Isolation and characterization of new Saccharomyces cerevisiae mutants perturbed in nuclear pore complex assembly." BMC Genet 3: 17.

Ryan, K. J., Y. Zhou and S. R. Wente (2007). "The karyopherin Kap95 regulates nuclear pore complex assembly into intact nuclear envelopes in vivo." Mol Biol Cell 18(3): 886-898.

Santos-Rosa, H., H. Moreno, G. Simos, A. Segref, B. Fahrenkrog, N. Pante and E. Hurt (1998). "Nuclear mRNA export requires complex formation between Mex67p and Mtr2p at the nuclear pores." Mol Cell Biol 18(11): 6826-6838.

Shibata, Y., C. Voss, J. M. Rist, J. Hu, T. A. Rapoport, W. A. Prinz and G. K. Voeltz (2008). "The reticulon and DP1/Yop1p proteins form immobile oligomers in the tubular endoplasmic reticulum." J Biol Chem 283(27): 18892-18904.

Strambio-De-Castillia, C., M. Niepel and M. P. Rout (2010). "The nuclear pore complex: bridging nuclear transport and gene regulation." Nat Rev Mol Cell Biol 11(7): 490-501.

Tcheperegine, S. E., M. Marelli and R. W. Wozniak (1999). "Topology and functional domains of the yeast pore membrane protein Pom152p." J Biol Chem 274(8): 5252-5258.

Walther, T. C., A. Alves, H. Pickersgill, I. Loiodice, M. Hetzer, V. Galy, B. B. Hulsmann, T. Kocher, M. Wilm, T. Allen, I. W. Mattaj and V. Doye (2003). "The conserved Nup107-160 complex is critical for nuclear pore complex assembly." Cell 113(2): 195-206.

Walther, T. C., P. Askjaer, M. Gentzel, A. Habermann, G. Griffiths, M. Wilm, I. W. Mattaj and M. Hetzer (2003). "RanGTP mediates nuclear pore complex assembly." Nature 424(6949): 689-694.

Winey, M., D. Yarar, T. H. Giddings, Jr. and D. N. Mastronarde (1997). "Nuclear pore complex number and distribution throughout the Saccharomyces cerevisiae cell cycle by three-dimensional reconstruction from electron micrographs of nuclear envelopes." Mol Biol Cell 8(11): 2119-2132.

Witkin, K. L., J. M. Friederichs, O. Cohen-Fix and S. L. Jaspersen (2010). "Changes in the nuclear envelope environment affect spindle pole body duplication in Saccharomyces cerevisiae." Genetics 186(3): 867-883.